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Prevalence of *Salmonella enterica, Listeria monocytogenes,* and *Escherichia coli* Virulence Factors in Bulk Tank Milk and In-Line Filters from U.S. Dairies†

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ABSTRACT

The zoonotic bacteria *Salmonella enterica, Listeria monocytogenes,* and *Escherichia coli* are known to infect dairy cows while not always causing clinical signs of disease. These pathogens are sometimes found in raw milk, and human disease outbreaks due to these organisms have been associated with the consumption of raw milk or raw milk products. Bulk tank milk (BTM) samples (536) and in-line milk filters (519) collected from dairy farms across the United States during the National Animal Health Monitoring System's Dairy 2007 study were analyzed by real-time PCR for the presence of *S. enterica* and pathogenic forms of *E. coli* and by culture techniques for the presence of *L. monocytogenes*. *S. enterica* was detected in samples from 28.1% of the dairy operations, primarily in milk filters. *Salmonella* was isolated from 36 of 75 PCR-positive BTM samples and 105 of 174 PCR-positive filter samples, and the isolates were serotyped. Cerro, Kentucky, Muenster, Anatum, and Newport were the most common serotypes. *L. monocytogenes* was isolated from 7.1% of the dairy operations, and the 1/2a complex was the most common serotype, followed by 1/2b and 4b (lineage 1). Shiga toxin genes were detected in enrichments from 15.2% of the BTM samples and from 51.0% of the filters by real-time PCR. In most cases, the cycle threshold values for the PCR indicated that toxigenic strains were not a major part of the enrichment populations. These data confirm those from earlier studies showing significant contamination of BTM by zoonotic bacterial pathogens and that the consumption of raw milk and raw milk products presents a health risk.

The presence of zoonotic bacteria such as *Salmonella* spp., *Listeria monocytogenes*, and enterohemorrhagic *Escherichia coli* (EHEC) on dairy farms and in the feces of dairy cattle has been well documented *(4, 11, 17)*. Dairy cattle are natural reservoirs for these organisms, and although the clinical diseases salmonellosis and listeriosis do occur in cattle, *Salmonella*, *Listeria,* and EHEC are often shed in the feces of asymptomatic animals. Additionally, the environment of the dairy farm is conducive to contamination by and persistence of many bacterial species. Cows can be exposed through contaminated water, feed, contact with wildlife, and environmental contamination. Despite considerable efforts to establish hygienic milking systems and protocols, fecal contamination is inevitable, and therefore, milk is at risk for contamination with any pathogen that is present in the feces or farm environment.

Although prevalence estimates vary, *Salmonella* spp., *L. monocytogenes*, and EHEC contaminate raw, bulk tank milk (BTM), and therefore, the milk is a potential source for human exposure. Various surveys have determined the prevalence of BTM contamination with these pathogens. Contamination of bulk tank milk by *Salmonella* spp., *L. monocytogenes*, and Shiga toxin–producing *E. coli* ranged from 0.2 to 8.9%, 1.0 to 12.6%, and 0.8 to 3.8%, respectively, in regional and national surveys *(19, 23, 30, 33–35, 38, 43, 46, 54)*.

Pasteurization is an effective tool for eliminating bacterial pathogens in milk, so most consumers are at minimal risk of exposure from milk consumption. However, raw milk is still consumed by a large proportion of farm families and workers and by a growing segment of the general population who believe that the milk is not only safe but also imparts beneficial health effects that are destroyed by pasteurization *(22, 29, 60)*. Therefore, consumption of both raw milk and raw milk cheeses has frequently been associated with foodborne illness, most notably due to *Campylobacter* spp., EHEC, *Salmonella* spp., and *L. monocytogenes*. The outbreaks have been attributed to milk or product from both licensed and nonlicensed raw milk sales operations, and the number of documented cases per outbreak was generally less than 100 *(29, 37, 60)*.

Postpasteurization exposure in the dairy plant and improper pasteurization are also potential risks and have

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TABLE 1. *State of origin and summary of BTM samples and filter samples received between 5 March and 31 August 2007*

State	No. of operations sampled	Sample counts (no.)					
		Milk	Filter	Pairs[a]	Milk, no filters[b]	Filters, no milk[c]	Total[d]
CA	34	34	34	34	0	0	68
ID	26	26	26	26	0	0	52
IN	10	10	9	9	1	0	19
IA	38	38	37	37	1	0	75
KY	11	11	11	11	0	0	22
MI	35	35	34	34	1	0	69
MN	33	31	33	31	0	2	64
MO	14	14	12	12	2	0	26
NM	2	2	2	2	0	0	4
NY	50	50	49	49	1	0	99
OH	39	39	37	37	2	0	76
PA	60	60	59	59	1	0	119
TX	16	16	16	16	0	0	32
VT	29	29	29	29	0	0	58
VA	22	22	21	21	1	0	43
WA	20	20	20	20	0	0	40
WI	99	99	90	90	9	0	189
Total	538	536	519	517	19	2	1,055

[a] Number of operations from which there was both a BTM sample and an in-line filter sample.
[b] Number of operations from which milk samples were submitted but there was no corresponding filter sample from the same operation.
[c] Number of operations from which filter samples were submitted but there was no corresponding milk sample from the same operation.
[d] Total number of milk samples and filter samples.

been known to cause contamination of milk products and subsequent foodborne outbreaks *(16, 39)*. However, this is a relatively rare source of foodborne disease. According to the Centers for Disease Control and Prevention, there have only been three documented cases of human listeriosis in the United States where postpasteurization contamination was implicated *(10)*. A particularly notable outbreak occurred in Massachusetts in 2007 due to the consumption of *L. monocytogenes*–contaminated pasteurized milk *(10)*. Of the five cases that were identified in this outbreak, three adult men died, one infected woman delivered her baby prematurely, and a second infected woman delivered a stillborn baby. So, although the occurrence is rare, the potential consequences of such an outbreak can be severe. More recently, an outbreak of *Salmonella* Braenderup was linked to pasteurized milk (as well as juices) produced at a single plant in Oregon *(40)*. Twenty-three people were infected with the same strain of *Salmonella* Braenderup between October 2009 and October 2010, and matching strains were isolated from surfaces in the dairy plant. Apparently environmental contamination in the plant resulted in external contamination of milk and juice containers after they were filled with pasteurized product.

In recent years, there has been a great deal of discussion in the United States and Canada regarding the risks associated with raw milk consumption. The U.S. Food and Drug Administration bans interstate sales of raw milk, but some states allow raw milk to be sold either directly to the consumer or indirectly through cow share programs. In order to ascertain the risks associated with consumption of raw milk or raw milk products, estimates of the frequency with which the milk is contaminated with zoonotic bacteria are needed. The Animal and Plant Health Inspection Service designs surveys of farms that represent the national herds for five production species. Milk samples were collected from dairy farms in a 2002 study, and this was the first national survey of BTM. Sampling the national herd again in 2007 would provide an opportunity to detect major shifts in pathogen prevalence. Additionally, we have demonstrated the utility of testing in-line milk filters as a sensitive way to detect the presence of pathogens. Therefore, milk filter testing was conducted in the 2007 survey to more accurately determine the prevalence of these bacterial pathogens on dairy operations.

The objective of this study was to determine the current prevalence of *Salmonella* spp., *L. monocytogenes*, and pathogenic *E. coli* in raw, BTM, and milk filters from throughout the United States.

MATERIALS AND METHODS

Samples. Samples used in this study were collected during the National Animal Health Monitoring System's (NAHMS) Dairy 2007 survey *(52)*. Data from the U.S. Department of Agriculture's National Agricultural Statistics Service were used to select a stratified random sample based on herd size from each of 17 participating states, representing 79.5% of dairy herds and 82.5% of dairy cows in the United States (California, Idaho, Indiana, Iowa, Kentucky, Michigan, Minnesota, Missouri, New Mexico, New York, Ohio, Pennsylvania, Texas, Vermont, Virginia, Washington, and Wisconsin) (Table 1). The survey design was a stratified random sample with unequal selection probabilities. The selection probabilities were unequal to ensure the inclusion of large dairy operations. All respondent data were statistically weighted to ensure that samples reflected the subject population. Complete details for the sample weighting and study design are available *(52, 53)*.

Those producers reporting one or more milk cows in inventory on 1 January 2007 were included in Phase I of the NAHMS Dairy 2007 study. In Phase I, National Agricultural Statistics Service enumerators administered a general management questionnaire. For Phase II data collection, which included BTM and milk filter sampling, operations with 30 or more milk cows on 1 January 2007 that participated in Phase I and agreed to continue participating were sampled by federal and state veterinary medical officers or animal health technicians. Samples were collected from March to August of 2007. BTM (50 ml) and in-line milk filters were aseptically collected from dairy operations and shipped overnight with cold packs to the U.S. Department of Agriculture, Agricultural Research Service laboratory in Beltsville, MD. Upon arrival in the laboratory, samples were immediately partitioned for analyses.

Bacteriological methods. For enrichment of *Salmonella*, 10 ml of milk was added to 10 ml of double-strength tetrathionate broth (BD Diagnostics, Sparks, MD). In-line milk filters were cut into small (30 to 50 cm^2) pieces and placed in a filtered stomacher bag, diluted (1 to 1 [wt/wt]) with 1% buffered peptone water, and pummeled in an automatic bag mixer for 2 min. The bag was removed from the mixer, filter pieces were repositioned to the bottom of the bag, and the bag was pummeled for 2 additional minutes. For enrichment of *Salmonella*, 20 ml of filtrate was added to 20 ml of double-strength tetrathionate broth. For all samples, enrichment tubes were incubated at 37°C for 18 to 24 h, after which 2 ml of the enrichment broth was centrifuged (16,000 × *g*), and the supernatants were discarded. The bacterial pellets were suspended in 0.5 ml of 1× freezing medium for cells of Schleif and Wensink *(45),* and the samples were stored at −80°C. Additionally, 1.5 ml of the enrichment culture was centrifuged (16,000 × *g*), and nucleic acids were extracted from the bacterial pellets using 200 μl of a commercially prepared extraction preparation (InstaGene Matrix, Bio-Rad Laboratories, Hercules, CA) following the manufacturer's directions. The DNA preparations were stored at −20°C prior to analysis by real-time PCR.

Real-time PCR was performed using the primers developed by Rahn et al. *(42)* that were shown by Malorny et al. *(31)* to be highly specific for *Salmonella* yet able to detect a wide range of serotypes. The PCR reaction was rendered real time by the addition of a fluorescent intercalating dye (EvaGreen, Biotium, Inc., Hayward, CA). A master mix for 100 reactions consisted of 300 μl of polymerase mix (see below), 1,700 μl of water, 300 μl of 10× buffer containing bovine serum albumin (BSA) and 20 mM $MgCl_2$ (Idaho Technology, Inc., Salt Lake City, UT), 300 μl of 10× deoxynucleoside triphosphate mix (Idaho Technology), 90 μl of 10× BSA (2.5 mg/ml, Idaho Technology), 150 μl of 20× EvaGreen dye in PBS, 15 μl of a 10 μM solution of each primer, and 30 μl of a 1:300 dilution of 6-carboxy-X-rhodamine (ROX) reference dye (Stratagene, La Jolla, CA) for a total of 2,900 μl. The polymerase mix consisted of 12 μl of Klentaq polymerase (AB Peptides, St. Louis, MO), 12 μl of TaqStart antibody (Invitrogen Corp., Carlsbad, CA); the components were mixed and allowed to sit at room temperature for 10 minutes and then diluted with 280 μl of enzyme diluent (Idaho Technology). Each reaction mixture consisted of 29 μl of PCR master mix and 1 μl of sample.

PCR was run on a Stratagene Mx4000 instrument (Stratagene, La Jolla, CA) with a thermal profile of 3 min at 94°C followed by 40 cycles of 94°C for 6 s, 67°C for 10 s, 72°C for 15 s, and 85°C for 35 s. The fluorescence in the 6-carboxyfluorescein (FAM) and ROX channels was read during the stringent 85°C plateau. Following amplification, a melting curve was run from 55 to 95°C in 1-degree increments. The fluorescence threshold for determining the cycle threshold (Ct) for positive samples was set manually for each run. Samples that showed a Ct of less than 40 cycles and a melt peak within ±1°C of that of the *Salmonella enterica* Montevideo positive control were considered positive for *Salmonella*.

When the PCR analysis indicated the presence of *Salmonella*, preserved enrichment biomass (10 μl) was streaked onto XLT4 agar (XLT4 agar base with XLT4 supplement, BD Diagnostics, Sparks, MD). Plates were incubated at 37°C and scored at 24 and 48 h for presumptive *Salmonella* (black colonies). Isolated presumptive *Salmonella* colonies were transferred from XLT4 plates onto XLT4, brilliant green, and L-agar (Lennox Broth base with 1.5% agar; Gibco Laboratories, Long Island, NY) and incubated at 37°C for 24 h. Colonies that exhibited the *Salmonella* phenotype (black on XLT4 and pink on brilliant green) were preserved from the L-agar for future analysis. Colony biomass was transferred from the L-agar plates to a vial containing 0.5 ml of 1× freezing medium for cells *(45),* and the samples were stored at −80°C. L-Agar slants were inoculated and, after incubation at 37°C for 24 h, sent to the National Veterinary Services Laboratories in Ames, IA, for serotyping.

For enrichment of *Listeria*, 10 ml of milk was added to 10 ml of double-strength modified *Listeria* enrichment broth (MLEB; BD Diagnostics, Sparks, MD). For in-line milk filters, 20 ml of filtrate from the pummeled filter pieces–peptone water mixture was added to 20 ml of double-strength modified *Listeria* enrichment broth. Enrichment tubes were incubated at 37°C for 48 h, after which 2 ml of the enrichment broth was centrifuged (16,000 × *g*) and the supernatants were discarded. The pelleted biomass was suspended in 0.5 ml of 1× freezing medium for cells *(45),* and the samples were stored at −80°C. Additionally, the broth was streaked (10 μl) onto modified Oxford medium (MOX) agar (Difco Laboratories, Detroit, MI). The MOX was supplemented with 50 μg/ml cycloheximide to inhibit fungal growth. Plates were incubated at 37°C and scored at 24 and 48 h for presumptive *Listeria* colonies. Isolated presumptive *Listeria* colonies were transferred from MOX plates supplemented with 50 μg/ml cycloheximide onto MOX, PALCAM (polymyxin acriflavin lithium-chloride ceftazidime esculin mannitol; BD Diagnostics, Sparks, MD), Trypticase soy agar with 0.6% yeast extract, and a chromogenic plating medium, BCM *Listeria* (Biosynth International, Inc., Naperville, IL). Colonies that exhibited the *Listeria* phenotype (esculin hydrolysis, flat, silvery, sometimes-dimpled colonies on MOX, and gray-green with esculin hydrolysis on PALCAM) were preserved for future analysis. Colony biomass was transferred from the PALCAM plates to 1.5 ml of tryptic soy broth and incubated at 37°C for 48 h. The culture was centrifuged (16,000 × *g*), and the supernatants were discarded. Bacterial pellets were suspended in 0.5 ml of 1× freezing medium for cells *(45),* and the isolates were stored at −80°C. The hemolytic activity of select presumptive *L. monocytogenes* isolates (blue colonies on BCM *Listeria*) was determined by stabbing blood agar (Columbia with 5% sheep blood; Remel, Lenexa, KS) and incubating at 37°C for 48 h. The Christie-Atkins-Munch-Peterson test was performed on selected isolates using *Staphylococcus aureus* beta lysin disks (Remel) and *Rhodococcus equi* (ATCC 6939, American Type Culture Collection, Manassas, VA) on sheep blood agar.

The serogroups of *L. monocytogenes* isolates were determined using a modification of the PCR method of Doumith et al. *(12)* wherein all conditions remained the same except that AmpliTaq Gold polymerase and buffer (Applied Biosystems, Foster City, CA) were used, necessitating the addition of a 10-min enzyme activation step to the start of the thermal program. Under these conditions, the 5-plex described by Doumith et al. *(12)* did not work consistently in our laboratory, so the reaction mixtures were

split into a 3-plex of *prs, lmo0737,* and ORF2819 and a duplex of *lmo1118* and ORF2110. The lineage of *L. monocytogenes* isolates that did not fall into the 1/2a, 1/2b, 1/2c, or 4b complexes of Doumith were determined using the PCR method of Ward et al. *(57). L. monocytogenes* strains of known serotype and lineage were obtained from the Agricultural Research Service (NRRL) Culture Collection for use as standards.

For enrichment of *E. coli*, 10 ml of milk was added to 10 ml of double-strength EC broth (Difco Laboratories, Detroit, MI). For in-line milk filters, 20 ml of filtrate from the pummeled filter pieces–peptone water mixture was added to 20 ml of double-strength EC broth. The enrichment tubes were incubated at 37°C for 24 h, after which 2 ml of the enrichment broth was centrifuged (16,000 × *g*) and the supernatants were discarded. The pelleted biomass was suspended in 0.5 ml of 1× freezing medium for cells *(45),* and the samples were stored at −80°C. A second aliquot of enriched broth (1.5 ml) was centrifuged (16,000 × *g*), and the supernatants were discarded. DNA was extracted from the bacterial pellets using a commercially prepared extraction preparation (InstaGene Matrix, Bio-Rad Laboratories, Hercules, CA) following the manufacturer's directions. The DNA preparations (200 μl) were stored at −20°C prior to analysis.

Two multiplex TaqMan real-time PCR assays were used to analyze the DNA extracts for the presence of Shiga toxin genes 1 and 2 (stx_1 and stx_2), generic intimin (*eaeA*), and the γ-allele of the translocated intimin receptor (*γ-tir*). The first reaction mixture consisted of an internal amplification control targeting a region of the *N*-methylcarbamate hydrolase gene (*mcd*, GenBank sequence accession no. AF160188) using primers mcd475f (CTAGAGCTCGCTGGCTTGAAG) and mcd475r (GATCTG-ACCGATTGTCGCG) and the 6-carboxyfluorescein–black hole quencher-1 (FAM-BHQ1)–labeled probe mcd475FAM (TCGAG-GTGGTTCCCCTTCCGG). DNA for the internal amplification control was amplified from plasmid pJK340 *(48)* using primers mcd475BIGf (ACGATTTGCAGCTTTGATTCG) and mcd475BIGr (ACCATGGCGATCCCGTC) to generate a 283-bp fragment that contains the 63-bp target of the real-time reaction. The purified PCR product was added to real-time reaction mixtures in an amount empirically determined to give a Ct value of between 32 and 35 cycles. The internal amplification control was duplexed with the real-time assay for stx_1 described by Ibekwe et al. *(21)* with a CY5–black hole quencher-2 labeled probe. The second multiplex PCR reaction targeted stx_2, eaeA, and *γ-tir* using primers and probes described previously *(25),* with the stx_2, *eaeA,* and *γ-tir* probes labeled with 6-carboxyfluorescein–black hole quencher-1, CY5-black hole quencher-2, and 6-carboxy-2′,4,4′,5′,7,7′-hexachlorofluorescein (HEX)–black hole quencher-1, respectively. Primer (300 nM) and probe (250 nM) concentrations were the same for each target and were added, along with BSA (final concentration 50 μg/ml), to 25 μl of real-time PCR master mix with low ROX (Eurogentec, Inc., San Diego, CA) to give a final volume of 48 μl. Two microliters of extracted sample DNA was added to each reaction mixture. Extracted DNA from *E. coli* O157:H7 SEA13B-88 (Odwalla strain, stx_1, stx_2, *eaeA,* and *γ-tir* positive) was used as a positive control. The thermocycler program consisted of a 10-min plateau at 95°C to activate the enzyme followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Fluorescence was determined during the 60°C step. The fluorescence threshold was set manually for each dye at the end of a run, and the Ct values were determined for each gene. Samples showing Ct values of fewer than 40 cycles were considered positive. The internal amplification control did not fail to amplify in any sample derived from the milk filters; however, it did fail in 3 milk-derived samples, and these latter samples were not included in the statistical analysis.

Statistical analysis. Statistical analysis incorporated operation weights to allow the samples to reflect the population from which they were selected (dairy cows on U.S. dairy operations with 30 or more cows in the 17 states included in the study). The weighting procedure adjusts for each operation's probability of selection during sampling and accounts for survey nonresponse. The weighted prevalence (WP) of the population also allows for more accurately evaluating trends over time. A commercially available software program (SUDAAN, release 8, Research Triangle Institute, Research Triangle Park, NC) was used to account for the complex and weighted study design. Due to the relatively small number of positive samples, serotypes are presented as unweighted estimates.

RESULTS

A total of 536 BTM samples and 519 milk filter samples were received between March 5 and August 31, 2007 (Table 1). Paired samples (milk and filter) were received from 517 dairy operations, while 19 operations were only represented by a milk sample, and 2 operations were only represented by a filter sample. Duplicate paired samples were received from four operations and a pair plus an extra milk sample were received from one operation. Overall, samples were received from 538 operations in 17 states.

Weighted prevalence estimates are presented in the paper. Unweighted estimates, or straight percentages, could be biased because operations did not participate at the same level for each herd size and region category. The weighting of operations allows operations that did not participate to be represented by similar operations that were sampled.

When DNA extracts from enriched cultures were analyzed by real-time PCR, 75 milk samples and 174 filters were positive for the presence of *Salmonella* (Table 2). These PCR-positive samples were from 197 dairy operations (28.1%). When a sample was identified as PCR positive, the preserved enrichment was cultured for the isolation of *Salmonella*. *Salmonella* isolates were obtained from 36 BTM samples and 105 filters. Based on the combined BTM sample and filter results, *Salmonella* was isolated from 12.7% (WP) of the operations. Isolates were obtained from both the BTM and milk filter from 30 operations; isolates were obtained from filters of 74 operations where the matching milk sample was culture negative and from the milk of 6 operations where the matching filter sample was culture negative. Five of these culture-negative filter samples were PCR positive.

When available, a minimum of two *Salmonella* isolates per sample were serotyped, and 336 isolates were serotyped in total. Twenty-two different serotypes were identified, and one isolate was untypeable (Table 3). Cerro was the most frequently isolated serotype, and it was isolated from 23.6% (unweighted estimate) of the *Salmonella*-positive samples. Serotypes Kentucky, Muenster, Anatum, and Newport represented 14.2, 10.1, 8.1, and 6.7% of the positive samples, respectively. Multiple serotypes were detected in six of the positive samples, and these were all from filters.

When the BTM samples and filters were cultured for the presence of *Listeria* spp., isolates were obtained from 43 BTM samples and 139 filters (Table 4). *L. monocytogenes* isolates were obtained from 24 BTM samples and 34 filters.

TABLE 2. *Number and weighted percent of operations that tested positive for* Salmonella *by PCR and culture*[a]

Sample type and result	Results by PCR		Results by culture	
	No. of operations	Weighted % of operations (SE)	No. of operations	Weighted % of operations (SE)
Positive milk	75	10.8 (1.8)	36	4.3 (1.0)
Positive filter	174	24.7 (2.4)	105	12.4 (1.6)
Positive milk and positive filter	52	5.9 (1.2)	30	3.0 (0.8)
Negative milk and positive filter	122	18.5 (2.2)	74	9.3 (1.5)
Positive milk and negative filter	22	5.1 (1.5)	6	1.3 (0.7)
Operations with any positive sample	197	28.1 (2.6)	111	12.7 (1.6)

[a] Selection of operations was not random to ensure the inclusion of large dairy operations. Weighting was necessary to adjust for each operation's probability of selection during sampling and to adjust (postsampling) for nonresponse.

Both the BTM sample and the filter were positive for *L. monocytogenes* in samples from 11 operations, whereas the paired BTM samples from 23 positive filters were negative, and the paired filter samples from 13 positive BTM samples were negative. *L. monocytogenes* was isolated from at least one sample from a total of 47 operations (7.1% WP).

Based on the serogrouping method of Doumith et al. *(12),* 46.6% (27) of the *L. monocytogenes* isolates were serotype 1/2a, 27.6% (16) were serotype 1/2b, and 22.4% (13) were serogroup 4b. The serotype could not be determined for seven isolates using the Doumith method. Therefore the PCR-based method of lineage identification described by Ward et al. *(57)* was used to determine that four of the isolates were in lineage 3 and were probably serotype 4b. Neither lineage or serotype could be determined for the remaining isolates. Three *L. moncytogenes* isolates from each of 46 samples were serotyped. From the remaining positive samples, only two isolates were available for serotyping from seven samples, and only one isolate was available from five samples. Multiple serotypes were

TABLE 3. Salmonella *serotype distribution in BTM samples, filter samples, and in milk and filter sample pairs*

Serotype	Milk[a]		Filter[b]		Milk/filter pairs[c]		Total samples		Total operations	
	No.	%[d]	No.	%	No.	%	No.	%	No.	%
Agona	0	0	0	0	1	4.2	2	1.4	1	0.8
Anatum[e]	2	16.7	6	6.8	2	8.3	12	8.1	10	8.1
Cerro	3	25.0	22	25.0	5	20.8	35	23.6	30	24.2
Derby	0	0	1	1.1	0	0	1	0.7	1	0.8
Dublin	1	8.3	3	3.4	1	4.2	6	4.0	5	4.0
Give[f]	0	0	3	3.4	0	0	3	2.0	3	2.4
Infantis	1	8.3	1	1.1	0	0	2	1.4	2	1.6
Kentucky	1	8.3	12	13.6	4	16.7	21	14.2	17	13.7
Mbdanka	1	8.3	3	3.4	2	8.3	8	5.4	6	4.8
Meleagridis	0	0	5	5.7	1	4.2	7	4.7	6	4.8
Montevideo	2	16.7	7	8.0	0	0	9	6.1	9	7.3
Muenchen	0	0	1	1.1	0	0	1	0.7	1	0.8
Muenster	1	8.3	6	6.8	4	16.7	15	10.1	11	8.9
Newport	0	0	8	9.1	1	4.2	10	6.7	9	7.3
Reading	0	0	1	1.1	0	0	1	0.7	1	0.8
Saint-Paul	0	0	1	1.1	0	0	1	0.7	1	0.8
Schwarzengrund	0	0	0	0	1	4.2	2	1.4	1	0.8
Senftenberg	0	0	1	1.1	1	4.2	3	2.0	2	1.6
Soerenga	0	0	1	1.1	0	0	1	0.7	1	0.8
Thompson	0	0	1	1.1	0	0	1	0.7	1	0.8
Typhimurium	0	0	3	3.4	1	4.2	5	3.4	4	3.2
3,10, poorly motile	0	0	1	1.1	0	0	1	0.7	1	0.8
Untypeable	0	0	1	1.1	0	0	1	0.7	1	0.8
Total	12	99.9	88	99.6	24	100.2	148	100.1	124	99.9

[a] Number of operations on which the serotype was found only in a milk sample.
[b] Number of operations on which the serotype was found only in a filter sample.
[c] Number of operations on which the serotype was found in both the milk and filter samples.
[d] Percentage of total serotypes found in the respective samples.
[e] Anatum grouping includes Anatum var. 15+, 34+ (one isolate) and Anatum var. 15+ (one isolate).
[f] Give grouping includes Give var. 15+, 34+ (one isolate) and Give var. 15+ (one isolate).

TABLE 4. *Number and weighted percent of operations from which* Listeria *organisms were isolated from milk and filter samples*[a]

Sample type and result	*Listeria* spp.		*Listeria monocytogenes*	
	No. of operations	Weighted % of operations (SE)	No. of operations	Weighted % of operations (SE)
Positive milk	43	9.0 (1.9)	24	3.7 (1.2)
Positive filter	139	28.3 (2.9)	34	5.1 (1.2)
Positive milk and positive filter	23	3.3 (0.9)	11	1.4 (0.6)
Negative milk and positive filter	114	24.5 (2.7)	23	3.6 (1.1)
Positive milk and negative filter	20	6.1 (1.8)	13	2.5 (1.1)
Operations with any positive sample	159	32.1 (2.8)	47	7.1 (1.5)

[a] Selection of operations was not random to ensure the inclusion of large dairy operations. Weighting was necessary to adjust for each operation's probability of selection during sampling and to adjust (postsampling) for nonresponse.

obtained from six filter samples and 1 BTM sample, and these samples represented five operations.

Shiga toxin genes (stx_1 and/or stx_2) were detected in 78 (15.2% WP) of the BTM samples tested, while *eaeA* was detected in 104 (16.1% WP) of the BTM samples (Table 5). In contrast, virulence genes were detected much more frequently in the milk filter samples; 302 milk filters (51.0% WP) were positive for Shiga toxin genes and 385 (64.8% WP) were positive for *eaeA*. The combination of genes associated with O157:H7 (stx_2, *eaeA,* and *γ-tir*) was detected in only 1.1% (5) of BTM samples, but it was detected in 6.3% (50) of the filters.

In BTM, the mean unweighted Ct values for stx_1, stx_2, *eaeA*, and *γ-tir* were 32.1, 31.1, 29.6, and 33.2, respectively. This indicates that, while the virulence factors were present in some members of the total population of bacteria that grew in the enrichment, those bacteria were not major members of the starting population. A similar result was seen in milk filter enrichments, where the mean Ct values for stx_1, stx_2, *eaeA*, and *γ-tir* were 32.1, 30.6, 28.4, and 32.5, respectively. For the samples that were stx_2, *eaeA*, and *γ-tir* positive, the Ct values also indicated the presence of very low numbers of potential EHEC in the starting enrichment population.

Only 14 samples (BTM or filter) from 13 operations were PCR positive for both *Salmonella* and *L. monocytogenes*. *Salmonella* was isolated from nine of these samples. Eight of the PCR *Salmonella*-positive, *L. monocytogenes*–positive samples were positive for at least one of the Shiga toxin genes, and *eaeA* was detected in 10 of these samples. The combination of stx_2, *eaeA,* and *γ-tir* was found in just one of the PCR *Salmonella*-positive and *L. monocytogenes*–positive samples.

DISCUSSION

The NAHMS of the Animal and Plant Health Inspection Service conducts national studies on the health and health management of United States domestic livestock and poultry populations. Surveys of the national dairy herd were conducted in 1996, 2002, and again in 2007. The results of the NAHMS 1996 study indicated that *E. coli* O157 and *Salmonella* were being shed by 0.9 and 5.4% of the milk cows tested and that, overall, 24.2 and 27.5% of the dairy operations had at least one cow (milk and/or cull) shedding *E. coli* O157 or *Salmonella,* respectively *(2, 49–51, 58).* To determine the impact that this level of exposure and shedding of pathogenic bacteria has on bulk milk contamination, the NAHMS 2002 survey included testing of raw BTM from each of the study farms. The results of the NAHMS Dairy 2002 study indicated that 3.8% (WP) of the raw milk samples were contaminated with *L. monocytogenes,* and salmonellae were isolated from 1.7% (WP) of the milk samples. Based on PCR analysis, the actual prevalence of *S. enterica* in U.S. BTM was much higher (11.8%) *(24).* Additionally, 23.2% of the raw milk samples contained potentially pathogenic forms of *E. coli,* and up to 4.2% contained a combination of virulence factors in their *E. coli* populations that was indicative of the presence of O157:H7.

Recent work has indicated that analysis of in-line milk filters is a more sensitive herd-level screening method for

TABLE 5. *Number and weighted percent of operations testing positive for* E. coli *virulence factors*[a]

Virulence factor(s)	Milk (n = 533)		Milk filter (n = 519)	
	No. of operations	Weighted % of operations (SE)	No. of operations	Weighted % of operations (SE)
stx positive	78	15.2 (2.3)	302	51.0 (3.0)
stx and *eaeA* positive	30	5.1 (1.4)	265	41.5 (2.9)
stx 2, *eaeA,* and *γ-tir* positive	5	1.1 (0.9)	50	6.3 (1.3)
eaeA positive	104	16.1 (2.2)	385	64.8 (2.9)
None	366	71.1 (2.7)	94	24.9 (2.7)

[a] Selection of operations was not random to ensure the inclusion of large dairy operations. Weighting was necessary to adjust for each operation's probability of selection during sampling and to adjust (postsampling) for nonresponse.

bacterial contaminants in the milk than testing of BTM itself *(28, 55)*. In-line filters are used to screen large particles from the milk as it is entering the bulk tank. The filters are not designed to trap microscopic particles, but it appears that bacteria get trapped along with the larger debris and, perhaps, some milk components. Additionally, the filters are a convenient sample to collect and ship. Therefore, the 2007 Dairy study was designed to include the collection of milk filters along with BTM.

Based on results from both the culture analysis and the PCR analysis, the prevalence of *Salmonella* contamination of bulk milk in the United States in 2007 was not different from that in 2002 *(24, 54)*. The level of contamination in each sample is not known since bacterial concentrations were not determined in either of these studies due to the increased labor needs for quantitative analysis. As anticipated, *Salmonella* was detected in a higher percentage of the milk filters than in BTM samples from the same operations. Isolates were obtained from 3 times more filters than milk samples, and PCR analyses detected *Salmonella* in 2.4 times more filters than BTM samples. Combining the results from the milk and filter analysis indicates that *Salmonella* was present on 12.7% (WP) of U.S. dairy operations, and PCR analysis indicated an even greater presence of this bacterium, since it was detected in samples from 28.1% (WP) of the operations. These *Salmonella* prevalence estimates concur with the fecal analysis results in the NAHMS Dairy studies in 1996 and 2002. Based on fecal analysis, these studies indicated that 27 to 31% of the herds had at least one animal that was shedding *Salmonella (2)*. An even higher percentage of *Salmonella*-positive farms was identified in a 16-herd study from four states *(5)*, where *Salmonella* was isolated from 10% of fecal samples (n = 960) and these positive isolates represented 9 of the 16 herds (56%).

S. enterica is a very diverse species, and more than 2,500 serotypes have been identified. There appears to be significant variability in virulence and infectious dose; however, all *Salmonella* serotypes are considered pathogenic to humans. Many different serotypes of *S. enterica* have been isolated from dairy animals and their environment, some of which represent significant serotypes in human clinical cases *(2, 5, 13, 54, 58)*. In the NAHMS Dairy 2002 study, nine *S. enterica* serotypes were identified in BTM, with Montevideo and Newport being the most common *(54)*. A greater diversity of serotypes (23) was isolated in the 2007 study, possibly due in part to the increased sensitivity of detection associated with testing the milk filters in addition to the BTM. However, the profile of serotypes was markedly different between the two studies. Although Montevideo was still one of the prominent serotypes, it only represented 6.1% of the *Salmonella* isolates in 2007, compared with 32% in 2002 (7 of 22 isolates). In 2007, serotypes Cerro and Kentucky were isolated most frequently, followed by Muenster and Anatum. Based on the 2006 *Salmonella* Annual Summary from the Centers for Disease Control and Prevention *(7)*, the serotypes that were identified in the BTM and milk filters have all been reported previously from clinical and nonclinical bovine samples, with the exception that there were no nonclinical serotype Soerenga isolates reported during 2006. Even though many of the identified serotypes are not frequently seen in humans, 8 of the top 20 serotypes most frequently reported to the Centers for Disease Control and Prevention from human sources in 2006 were isolated from BTM and/or milk filters in this study.

The likely source of salmonellae in the BTM is fecal contamination and, even with good hygienic practices during milking, it is difficult to keep all fecal material out of BTM. Many animals shed *Salmonella* in their feces but are asymptomatic. For example, in a 5-year study of a 100-cow dairy farm in PA, *Salmonella* was isolated from individual fecal samples and within-herd prevalence ranged from 8 to 88% *(41, 55, 56)*. However, the cows did not exhibit signs of salmonellosis and the producer would not have known that his cows were infected had his herd not been enrolled in the study. Throughout this period, *Salmonella* was routinely isolated from weekly samples of the bulk milk and milk filters, and the milk filters were found to be predictive of fecal prevalence *(55)*. When animals are asymptomatically shedding *Salmonella*, it is difficult to identify infected animals for specific precautions in the milking parlor, and therefore, the potential for bulk milk contamination is great.

L. monocytogenes is the significant *Listeria* species with respect to human disease. *L. monocytogenes* contamination of BTM and filter samples was detected in 47 U.S. dairy operations, indicating a significant risk (7.1% [WP]) of contamination not only for the purpose of raw milk and raw milk product consumption but also for the processing plants. *Listeria* spp. have been shown to be particularly persistent in production plants, primarily due to an ability to form biofilms *(1, 61)*; therefore, contamination of the raw milk entering the plant is a concern to the processors even when the milk is being pasteurized. Recent work has also suggested that *Listeria* can be present in biofilms that form in the milking parlor equipment, such as milk weight meters and stainless steel pipelines used to transfer milk from the milking units to the bulk tank *(27)*. Such a biofilm could represent a continuous source of bacteria in the milk tank, as outer cells are sloughed from the biofilm surfaces when milk passes through the pipeline system into the tank.

There are 13 known *L. monocytogenes* serotypes *(47)*, and 1/2a, 1/2b, and 4b are the most common serotypes among human clinical isolates. In the present study, all of the *L. monocytogenes* isolates, except for three isolates that were not typeable, were 1/2a, 1/2b, or 4b. Three of the 17 4b isolates were in lineage 3; isolates from this lineage appear to be better adapted to the animal environment rather than the food environment and, therefore, potentially pose less risk to humans *(57)*. Based solely on the serotyping information, 89% (56 of 63) of the *L. monocytogenes* strains isolated from the milk and milk filter samples were potential human pathogens.

These results are very similar to those obtained from the NAHMS Dairy 2002 study, where *L. monocytogenes* was isolated from 3.8% (weighted estimate) of the collected bulk milk samples and 93% of the isolates were serotypes 1/2a,

1/2b, and 4b *(54)*. Dairy farms have been identified as a reservoir of *L. monocytogenes,* and there is significant strain diversity within and across farms *(3, 4, 27, 28, 36)*. As with *Salmonella*, fecal shedding and environmental contamination of *L. monocytogenes* make contamination of the bulk milk very difficult to avoid. *L. monocytogenes* has also been cultured from the feces of healthy, asymptomatic dairy cattle, so identifying carriers is also difficult. In a recent survey of dairy farms in central New York State, Mohammed et al. *(32)* concluded that *L. monocytogenes* was isolated from dairy cattle or their environment more frequently than from the milk. As with other pathogens, hygiene is an important aspect of controlling the *L. monocytogenes* load in raw milk. In contrast with the multifarm survey, a longitudinal study of one New York State dairy farm demonstrated that *L. monocytogenes* was isolated from milk and in-line milk filters much more frequently than from the cows and their environment *(27, 28)*. In that case, it appeared that *L. monocytogenes*-containing biofilms were established in the milking system and acting as a consistent source of the bacterium in the bulk milk.

There are many different genotypes associated with pathogenic forms of *E. coli*. The forms associated with severe enterohemorrhagic disease (EHEC) generally produce Shiga toxins, encoded by *stx* genes, and the cell surface protein intimin, encoded by the *eaeA* gene. Less pathogenic strains may produce only Shiga toxin or only intimin. Those producing intimin without Shiga toxins (enteropathogenic *E. coli*) are frequently associated with infantile diarrhea. *E. coli* O157:H7, presently the EHEC most commonly isolated in the United States, produces Shiga toxin 2, intimin, and the γ form of the translocated intimin receptor; some strains may also produce Shiga toxin 1. Thus, the presence of any of these genes in the population of *E. coli* in BTM is cause for concern if the milk is to be consumed raw or used to make products without prior pasteurization.

The pathogenic *E. coli* results from this study cannot be directly compared with those from the NAHMS Dairy 2002 study because only *eaeA* was assayed in all samples in 2002 *(25)*. In 2002, *eaeA* was detected in 199/859 BTM samples (23.2%), which is similar to the prevalence observed in 2007 (19.5%). In the 2002 study, 36 samples contained *eaeA* and at least one of the *stx* genes, but only 2 of 859 samples met the PCR criteria for possible O157:H7 contamination (*eaeA, stx*$_2$, *fliC, hylA,* and *rfb*$_{O157}$). In the 2007 survey, 5 of 533 BTM samples met the somewhat less stringent criteria (*eaeA, stx*$_2$, and *γ-tir* positive) for O157:H7 contamination.

Based on the results of this study, it would appear that EHEC contamination of BTM is not a major public health concern in the United States. However, there have been outbreaks of foodborne illness traced back to the consumption of raw milk that was contaminated with *E. coli* O157:H7 *(8, 9, 26)*. There have been many reports of *E. coli* O157:H7 isolation from dairy farms, and so, contamination of the milk at some level is expected. Infrequent and/or low level contamination may not be picked up in a cross-sectional study such as this one, but such contamination can have serious public health consequences if the milk is not handled properly or not pasteurized before consumption.

Although only *Salmonella*, *L. monocytogenes*, and *E. coli* were the focus of this study, there are other notable zoonotic pathogens associated with milk and dairy products. In particular, *Campylobacter* spp. have also been identified as the cause of outbreaks associated with the consumption of milk and dairy products *(6, 15, 59)*. Based on several surveys *(14, 18, 44), Campylobacter* spp. are frequently shed by asymptomatic dairy cattle and, therefore, present challenges similar to those of *Salmonella*, *Listeria*, and *E. coli*.

Zoonotic bacteria, such as *Salmonella*, *Listeria*, and *E. coli*, as well as others that were not included in this study, are killed by pasteurization, and the majority of milk consumed in the United States is pasteurized. Therefore, for the most part, milk contaminated with these bacteria does not represent a large public health threat. However there is a significant risk of dietary exposure to bacterial pathogens associated with raw milk consumption. Many farm families drink raw milk *(20, 22),* and there is also a small but growing consumer interest in the consumption of raw milk. Although the U.S. Food and Drug Administration banned the interstate shipment of raw milk in 1987, raw milk can be sold legally in approximately half of the U.S. states. Some states allow the retail sale of raw milk, but most state legislation is more restrictive, allowing milk sales only at the farm level or under cow share programs. Although testing for specific pathogens may be a component of some state regulations, testing is often infrequent. For example, some regulations require raw milk sales permit holders to test for pathogens (*Salmonella, L. monocytogenes, Campylobacter,* and *E. coli* O157:H7) in order to obtain a permit and then again monthly or even less frequently. When an outbreak caused by the consumption of raw milk and raw milk products is traced to a farm source, consecutive tests with negative results are then typically required before sales are resumed.

As shown in this study and previous work, PCR analysis can provide a rapid and sensitive determination of the presence of *Salmonella*. However, the isolation of an organism is the gold standard for confirming the presence of the pathogen. Since DNA is the target of PCR analysis, DNA from nonviable as well as viable cells will result in a positive test. However, PCR analysis may also detect cells that are below the lower concentration limit of the culture method or cells that are viable but cannot compete with other bacterial strains in the culture medium. The use of PCR analysis in, for example, a testing program for raw milk sales would reduce the risk of contaminated milk reaching the consumer. However, it may also increase the loss of saleable raw milk. Perhaps a prescreening of the milk by PCR followed by an attempt to isolate the organism from PCR-positive samples would be a good approach. The advantages and disadvantages of using PCR analysis need to be further assessed and debated.

The ability to detect zoonotic bacteria in the BTM or milk filter from a contaminated herd is dependent on several on-farm factors, including the percentage of infected cows in the herd, the concentration of a specific pathogen being shed in the feces, and the attention paid to milking hygiene. If milk filters are a more sensitive indication of the bacteria

entering the bulk tank, the frequency of milk contamination is much higher than that detected by sampling milk alone.

When comparing sample types and testing methods, the prevalence of *Salmonella, E. coli,* and *Listeria* in milk was similar in 2002 and 2007. The addition of milk filter sampling, which appears to be more sensitive than sampling BTM, has increased the estimated prevalence of these organisms in the 2007 study. *Salmonella* or *L. monocytogenes* was detected in milk filters from about one-fourth of dairy operations in 2007. A regular, frequent testing program that includes milk filters and perhaps PCR analysis would be substantially more successful at identifying contaminated milk or milk products and minimizing public exposure to the zoonotic pathogens associated with dairy products.

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